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Pharmaceutical composition for suppressing undesired gene expression

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Pharmaceutical composition for suppressing undesired gene expression

Specification

The present invention is concerned with a possibility to suppress expression of undesired gene products, and especially with a pharmaceutical composition enabling such suppression of undesired gene products in the treatment or prevention of diseases associated with such gene expression, especially cancer diseases or conditions associated with viral infections.

Reverse genetics, a technique that proceeds from genotype to phenotype by gene-manipulation techniques, is an efficient strategy to assess the function of a gene. RNA interference (RNAi) has become an excellent approach for the analysis of gene function in invertebrates and plants (1-3). Fire and his colleagues demonstrated that in C. elegans the presence of just a few molecules of double-stranded RNA (dsRNA) was sufficient to reduce dramatically the expression of a gene that was homologues to the dsRNA (4). During this so-called process of RNA interference (RNAi) 21-25-nucleotide (nt) long RNAs known as short-interfering RNAs (siRNAs) were detectable that were complementary to both strands of the silenced gene and that were processed from a long dsRNA precursor (5-10). Genetic data on silencing through siRNAs revealed the involvement of an enzyme called Dicer (11), which contains two RNase III motifs, an RNA helicase domain and a dsRNA-binding domain. This enzyme seems to catalyse the processing of long dsRNA to 21-23-nt siRNA products (9;12). Amazing observations by Tuschl and colleagues revealed recently that transfection of synthetic 21-nt siRNA duplexes into mammalian cells efficiently inhibits endogenous gene expression in a sequence-specific manner (13). However, a significant disadvantage of siRNAs is that their effects are transient, with phenotypes generated by transfection with such

RNAs persisting for 1 week at most. Thus, several groups have sought to expand the utility of RNAi in mammalian cells by devising techniques to induce stable and heritable gene silencing. Different reports take advantage of RNA polymerase III promoters, which have well-defined initiation and termination sites producing various small RNA species. Beyond these, the U6 snRNA promoter and the H1 RNA promoter have been well characterized (14-16). A number of investigators was able to use this approach to demonstrate, in multiple mammalian cell lines, robust inhibition of several endogenous and exogenous genes of diverse functions (17-22). In our recent study the technique of RNA interference was used to define the role of polo-like kinase 1 (PLK1) in neoplastic proliferation (23). Expression of the PLK1 gene coding for a serine/threonine kinase that is highly conserved from yeasts to humans, is elevated in a broad range of human tumors (24). The importance of PLK1 as measure for the aggressiveness of a tumor seems to result from its leading role for mitotic progression in particular control of the G2/M transition (25;26). Moreover, PLK1 triggers also additional steps during mitotic progression: activity of the anaphase promoting complex and cytokinesis (27-29). All cancer cell lines (MCF-7 breast cancer cells, HeLa S3 cervical cancer cells, SW-480 colon cancer cells, and A549 lung cancer cells) transfected with low doses of siRNAs targeted to PLK1 had greatly decreased levels of PLK1 mRNA and protein. Cell proliferation was reduced and apoptosis increased in siRNA-treated cells. Transfected SW-480 cells were mitotically arrested and their centrosomes had lost the ability to nucleate microtubules. Interestingly, HMECs (human mammary epithelial cells) took up siRNAs less efficiently than cancer cells, and transfection with siRNAs targeted to PLK1 did not inhibit their proliferation. Thus, siRNAs targeted against human PLK1 may be valuable tools as antiproliferative agents that display activity against a broad spectrum of neoplastic cells at very low doses.

Considering the siRNA technology as a powerful strategy in reverse genetics, which exceeds the potential of antisense and Ribozyme

approaches, it is tempting to apply this technology to 'knock-down' the expression of genes in living animals. Recent studies describe methods for in vivo delivery of siRNAs to organs of adult mice and demonstrate inhibition of gene expression in various organs (2;30-32). Still, systemic treatment of tumor-bearing animals with siRNAs has not been investigated previously, but is of utmost interest, because the main cause of treatment failure and death of cancer is metastasis.

It is also of great interest to develop methods that allow the inhibition of viral infection of cells and the formation of viral progeny and expansion of the infection to other cells. Especially for viruses which cause severe diseases there is a vital desire to develop possibilities to either prevent infection after exposition to the virus or to stop the progression of the disease, thereby allowing the patient's immune system to defeat the virus efficiently.

For several other diseases, mechanisms of development of such diseases have been discussed that include elevated levels of certain gene products. Also such diseases are possible objectives for the present invention. As an example, overexpression of Interleukin 1 can be observed in Alzheimer's disease patients.

It was therefore an object of the present invention to develop corresponding treatments and pharmaceuticals that allow for effective and selective suppression of gene expression in vivo and that avoid drawbacks of the state of the art. It was especially desired to develop methods that can easily be applied and show a prolonged effectiveness as compared to already known gene silencing methods.

This object of the present invention was solved by providing pharmaceutical compositions and their uses for suppression of undesired gene expression.

A first subject of the present invention, therefore, is a pharmaceutical composition for suppressing gene expression comprising an effective amount of

1) an RNA expression system and

2) a nuclease inhibiting substance, wherein said RNA expression system contains

a) at least one RNA polymerase specific promoter sequence and under the transcriptional control of said promoter sequence

b) at least one genetic information homologous to said gene to be suppressed,

such genetic information being transcribed under suitable conditions and in the presence of an RNA polymerase into short interfering RNA, preferably small interfering double stranded hairpin RNA or short RNA antisense-strands (20-25 nt in lenght).

In the framework of the investigations leading to the present invention it was surprisingly found that an effective and persistent gene silencing is possible by way of addition of nuclease inhibiting substances to the RNA generating expression system. This nuclease inhibiting substances avoid the breakdown and removal of the expression vector containing the genetic information for the RNA and possibly also the RNA itself. Thus the expression vector in the presence of RNA polymerases which are abundant in vivo, can constitutively express RNAs targeted against the gene to be silenced for a sufficiently long time. It was found that application of the pharmaceutical composition of the present invention every other day was sufficient to suppress tumor growth and to allow immune systems to attack the tumors and thereby even reduce tumour size. Also it was observed that viral infections can be stopped or at least their effects be substantially reduced by administration of the present pharmaceutical composition.

The pharmaceutical composition of the present invention further showed no marked detrimental side effects to the treated person. To the contrary, the application can take place easily by for example intravenous injection of the composition.

The composition according to the invention contains an RNA expression system that either encodes siRNA, preferably small interfering hairpin RNA, or short antisense RNA, both of which are homologous to the gene to be expressed and interfere with proper transcription of said gene. The RNA expression system can contain one promoter and genetic information for one kind of interfering RNA, however, it is also possible that the composition contains an expression system containing genetic information for more than one kind of interfering RNA. In such case this genetic information for more than one kind of RNA can be expressed under the control of one promoter but also several promoters, being the same or different, can be used. The pharmaceutical composition of the present invention can also contain more than one expression system, each leading to formation of one or more kinds of interfering RNA.

The following investigations lead to the development of the present pharmaceutical compositions and their use in gene silencing:

Experimental introduction of siRNA duplexes 21-nt in length with 2 to 3 nt 3′ overhanging ends containing 5′ phosphate and free 3′ hydroxyl termini (10;13) into mammalian cells is now widely used to disrupt the activity of cellular genes homologous in sequence to the transfected siRNA. Previous studies in Drosophila and C. elegans demonstrated that hairpin dsRNA, which might be further processed by Dicer, silences also expression of a specific gene (33;34). Subsequent investigations took advantage of the human U6 snRNA promoter and its simple termination signal to express short hairpin RNAs (shRNAs) for efficient 'knock-down' of gene function. To generate shRNA targeted to PLK1 from a plasmid-based expression

system, we used a RNA PolIII-based strategy to synthesize transcripts terminating at a run of 4-5 thymidine nucleotides. This procedure warrants shRNAs with defined ends. We wished to test the possibility whether small endogenously encoded hairpin RNAs might also regulate human PLK1 in mammalian cells thereby suppressing proliferative activity as recently demonstrated for synthetic siRNAs targeted to PLK1 (23). Since our previous experiments showed that synthetical siRNA2 targeted to human PLK1 is most effective in suppressing PLK1 mRNA expression in HeLa S3 cells, we inserted corresponding annealed oligonucleotides that acted as templates for the synthesis of shRNAs into a plasmid containing the mouse U6 promoter that directs the synthesis of a PolIII-specific RNA transcript (18). The resulting hairpin RNA is composed of two complementary sequences 21 nt in length in an inverted orientation separated by a spacer of 6 nt in length (Fig. 1). To function as a termination signal for PolIII 5 thymidine nucleotides were hooked onto the 3'end. Transcription from the U6 promoter generate RNAs that are predicted to form hairpins as secondary structure with a 3' overhang of several thymidines. We used this approach to generate DNA templates for the synthesis of shRNAs corresponding to the recently used siRNA2 (shRNA/PLK1) and a scrambled version of siRNA2 (shRNA/PLK1S).

To find out whether this U6-driven approach is suitable to inhibit PLK1-expression in HeLa cells, we first performed Northern- and Western Blot analyses.

Although siRNAs are effective tools for inhibition of gene function in mammalian cells, their suppressive effects are by definition of limited duration. Thus, strategies are required that could bypass such limitations and provide a tool for evoking stable suppression by plasmid-driven expression of siRNAs or hairpin RNAs in mammalian cells. Nuclease activity (cellular and nucleases in the blood) is a potential barrier to the successful delivery of foreign DNA to and its function within mammalian cells. We

tested the hypothesis that transfection in the presence of specific nuclease inhibitors can enhance the expression of exogenous gene products. We used aurin tricarboxylic acid (ATA) to enhance the expression of U6-driven shRNAs in the human cell line HeLa S3. ATA has been shown previously to inhibit DNase I, RNase A, S1 nuclease, exonuclease III and various endonucleases (35;36). As shown by proton magnetic resonance spectroscopy the mechanism of action of ATA involves competition between the nucleic acid and the polymeric ATA for binding in the active site of a polypeptide such as nucleases (37).

Administration of synthetical siRNA or virus-mediated siRNA in adult mice decreased partially the expression of exogenous or endogenous genes (2;30-32). However, systemic administration of siRNA for the treatment of tumor-bearing animals had not been reported previously. Thus, the effects of shRNA on human tumor growth in vivo was next inspected using subcutaneously implanted tumor xenografts in nude mice. In these experiments recombinant U6 promoter-containing plasmids driving the expression of shRNAs (shRNA/PLK1, shRNA/PLK1S) in saline solution were administered to tumor-bearing mice by bolus intravenous injection every other day. For efficient delivery of plasmid DNA to organs of adult mice we injected rapidly a large volume of physiological solution into the tail vein of mice (38). According to this 'high-pressure' procedure a volume of 0.5-1.0 ml of saline containing plasmid pBS/U6/PLK1 or pBS/U6/PLK1S was injected. Since the stability of administered plasmids plays a critical role for the efficacy of shRNA expression, the stabilizing potential of ATA on plasmid DNA was tested in the blood of nude mice (Fig.2). While pure plasmid DNA was not detectable 2 hours after its intravenous injection into the tail vein of nude mice, a combination with ATA extended its live-time up 4-8 hrs. Thus, application of PBS containg plasmids and ATA was started three weeks following tumor fragment implantation, when tumors reached a volume of 50-100 mm^3. In initial experiments, shRNA/PLK1-expressing U6 plasmids were tested for 24 days at a dose of

0.25-0.4 mg/kg body weight in combination with 0.005-0.8mg ATA/kg body weight every other day and compared with the impact of shRNA/PLK1S-expressing plasmid containing the scrambled version of shRNA/PLK1 of as a control. shRNA/PLK1 displayed very potent antiproliferative effects on the growth of HeLa S3 tumors in mice, whereas tumor growth was not inhibited by the control shRNA/PLK1S (Fig. 3). During the entire experiment of 76 days mice treated with shRNA/PLK1 or shRNA/PLK1S at a dose of 0.33-0.4 mg/kg did not demonstrate decreased body weight. Interestingly, after termination of the therapy tumor size remained almost unchanged for a period of 4 weeks. From tumor-bearing mice which were treated for 28 days with shRNA/PLK1 or shRNA/PLK1S, total RNA was prepared from tumors and PLK1 levels were determined by Northern Blot analysis. Administration of shRNA/PLK1 to mice resulted in a complete suppression of PLK1 mRNA levels in tumors. In contrast, the frequency of PLK1 expression in mice treated with the scrambled control (shRNA/PLK1S) was not suppressed.

In summary, with the present invention we provide a powerful novel strategy to suppress very efficiently tumor cell proliferation in cell culture. For the first time it could be demonstrated that U6 promoter-driven hairpin RNAs targeted against PLK1 stabilized by the nuclease inhibitor ATA suppress tumor growth in nude mice when administered every other day systemically by intravenous injection. The power to encode a long-lasting silencing signal allows the combination of hairpin-mediated silencing with in vivo and gene delivery strategies for therapeutic approaches based on stable RNA interference in humans. Systemic RNA silencing in patients provides the fascinating perspective of using therapeutics that are of natural composition compared to chemical compounds, that are highly specific and that are of cost effective manufacturing. Our findings are the launch for the systemic treatment of diseases (cancer, viral infections such as AIDS etc.) which are characterized by undesirable gene expression.

In a preferred embodiment of the present invention, the RNA expression system contains a promoter sequence which is specific for class III RNA polymerases and especially preferrably it contains the U6 promoter (18). Using the U6 promoter in an expression system leads to a very stable expression of the corresponding siRNA or short RNA antisense strands and it is conceivable that nuclease inhibitor concentrations can even be lowered and possibly even nuclease inhibitors are not necessary at all for obtaining sufficient interfering RNA expression and corresponding gene silencing when using the U6 promoter.

The basic structure of the expression system is not critical as long as it allows for efficient transcription of the genetic information of component b) of the expression system. The expression can occur either constitutively or inducably. A constitutive expression is preferred in the present context. Usually a bacterial plasmid or a viral vector will form the basis of the expression system, however, the present invention is not limited thereto.

The preconditions for formation of siRNA are known to the person skilled in the art and can also be inferred from the references mentioned supra. The expression system contains two complementary and inverted DNA sequences which upon transcription by RNA polymerase lead to formation of double stranded RNA products. Such RNA products preferably are 15 to 30 nucleotides long and are homologous to the genes to be suppressed.

In a especially preferred embodiment of the present invention, the DNA coding for the siRNA is contained on a vector in the form of two complementary and inverted sequences which are adjacent to each other but divided by a spacer sequence, such spacer sequence being preferably 3 to 10 nucleotides long. Upon transcription of the expression system a small hairpin double stranded RNA (shRNA) is formed which interferes with expression of the gene to be silenced. It is however also possible to

provide for separate transcription of the two strands of the siRNA which will anneal after transcription automatically or of short RNA antisense strands.

In a preferred expression system on the 3'end of the sequences to be transcribed there is a RNA polymerase stop signal, preferably a T multimer.

As nuclease inhibitor every physiologically acceptable substance can be used that inhibits or decreases degradation of the expression system for a significant time. In a preferred embodiment of the present invention, as nuclease inhibitor aurin tricarboxylic acid (ATA) is used. However, the invention is not limited to this substance and every other nuclease inhibiting substance showing substantially similiar properties related to nuclease inhibiton and physiological tolerance is applicable.

It is a very favorable characterisitic of the pharmaceutical composition of the present invention that it can be applied easily, preferably by intravenous injection. In this context it is further preferred to include the pharmaceutical composition in a physiologically acceptable solution, e.g. phosphate buffered saline. Administration of larger volumes of solution promote the immediate effectiveness of gene suppression by the present pharmaceutical composition. Suitable amounts of administration of the expression system depend on the size of the plasmid or vector used. However, a preferred dosage of expression system including the sequences being transcribed to siRNAs lies between 0.05 to 0.5 mg/kg body weight of the patient. The effect of the generated siRNA can easily be monitored by assaying for remaining expression of the gene to be suppressed. Thus the dosage can easily be adjusted to the needs. Also the addition of further substances into the injection solution is possible. Such further substances can include symptom alleviating substances, substances that strenghen the patient, antibiotics or other suitable substances. Also substances that

facilitate application and carriers or auxiliaries can be included in the formulation.

In preferred embodiments of the present invention, the pharmaceutical composition is used for tumor therapy or prophylaxis. As described above and in more detail in the following examples, suppression of PLK1 expression has been shown to be an efficient method for treatment of cancer disease of several tissues. Also other proteins which are associated with tumor diseases like for example HER2/neu or BCR/ABL are promising candidates for gene silencing according to the present invention and therefore treatment or pevention of the corresponding cancer diseases.

In another preferred embodiment of the present invention, the pharmaceutical composition is targeted on viral genes that are actively involved in infection of cells or formation of virus progeny etc. Candidates to be combatted by the present invention are especially viruses for which no immunization is available and/or which are associated with grave diseases or damages to the patient. Examples for such viruses are Human Immunodeficiency Virus (HIV), Hepatitis viruses, Epstein Barr virus, Herpes simplex virus and Cytomegalo virus.

It is to be understood that the present invention does not only include the pharmaceutical composition described above but also the use of such composition for suppression of undesired gene expression as well as processes and methods for suppressing undesired gene expression using such combination of expression system for specific siRNA and nuclease inhibitor. Such processes and methods are very advantageous since they are very well tolerated, are not time consuming and the pharmaceutical composition can be produced easily and inexpensively.

Using the pharmaceutical composition according to the present invention or applying a corresponding method of treatment allows to inhibit tumor growth as is apparent from the examples. Using the present invention it is

also possible to avoid metastasis thus leading to either prolonged survival rates and better condition of patients since chemotherapy and other physical strain can be avoided.

The present invention is further illustrated by the following examples and figures.

Fig. 1 is a schematic representation of the promoter and sequences of the expression system which are coding for the siRNA targeted against PLK1.

Fig. 2 shows the stabilizing effect of ATA on DNA plasmids in murine blood.

Fig. 3 is a curve showing the effect of the invention on tumor growth.

Example

Materials and methods

Plasmids, sequences, antibodies, and nuclease inhibitor

Plasmids were constructed using standard techniques. We used the pBS/U6 vector containing sequence elements for cloning and bacterial replication. To generate first an intermediate plasmid for the subsequent cloning steps of hairpin RNAs targeted to PLK1, a 21-nt oligonucleotide (5'-GGCGGCTTTGCCAAGTGCTTA-3') annealed with an 25-nt oligonucleotide (5'-AGCTTAAGCACTTGGCAAAGCCGCC-3') corresponding to siRNA2 (23) was first inserted into the pBS/U6 vector, digested with ApaI (blunted) and HindIII. The inverted motif that contains the 6-nt spacer and a termination string of five thymidine residues (5'-AGCTTAAGCACTTGGCAAAGCCGCCCTTTTTTG-3', 5'-AATTCAAAAAGGGCGGCTTTGCCAAGTGCTTA-3') was then subcloned into the HindIII and EcoRI sites of the intermediate plasmid to generate pBS/U6/PLK1. Sequences of the hairpin RNAs targeted to PLK1

Monoclonal PLK1 antibodies for western blots were obtained from Transduction Laboratories (Heidelberg, Germany) and monoclonal antibodies against actin were obtained from Sigma-Aldrich (Taufkirchen, Germany). Goat anti-mouse secondary antibodies were purchased from Santa Cruz Biotechnology, Inc. (Heidelberg, Germany). Aurin tricarboxylic acid (ATA) was obtained from Sigma-Aldrich (Taufkirchen, Germany).

Cell Culture

Ham's F12 and fetal calf serum (FCS) were purchased from PAA Laboratories (Cölbe, Germany). Phosphate buffered saline (PBS), Opti-MEM I, glutamine, penicillin/streptomycin, and trypsin were from Invitrogen (Karlsruhe, Germany). FuGENETM6 was from Roche (Mannheim, Germany). The tumor cell line HeLa S3 (cervix) was obtained from DSMZ (Braunschweig, Germany) and cultivation was performed according to the supplier's instructions.

In vitro transfection with plasmids pBS/U6/PLK1, pBS/PLK1S and pBS/U6 HeLa S3 cells were transfected with plasmids according to the FuGENETM6 protocol (Roche, Mannheim, Germany). In brief, 1 day prior to transfection cells were seeded without antibiotics in a density of $2x10^5$ cells per 10 cm^2 cell culture dish (for determination of the growth rate over a period of 10 days cells were seeded in a density of $5x10^4$ cells per 10 cm^2 cell culture dish), corresponding to a density of approximately 50% at the time of transfection. Amounts of plasmids range between 0.5 and 6 μg plasmid per 10 cm^2 culture dish. Cells incubated with culture medium alone without plasmid were considered mock-treated cells. In the presence of the transfection reagent FuGENETM6 DNA delivery was performed using aurintricarboxylic acid (ATA) at a ratio of 1:5 (ATA:DNA) in order to stabilize plasmids and/or siRNAs. For this purpose plasmids and ATA were mixed and then added to the Opti-MEM I/FuGENETM6 mix. Just before transfection cells were covered with normal culture medium and the transfection mix (containing Opti-MEM I, FuGENETM6, and plasmids, with

or without ATA) was added. Following incubation of cells at 37°C for at least 4 hr to over night, fresh culture medium was added to a final volume of 2 ml/10 cm^2. Cells were harvested 6, 24, 48, 72, and 96 hr after the beginning of the transfection period for the analysis of mRNA or protein expression, DNA staining, and fluorescence activated cell sorting (FACScan) analysis. In addition, the stability of plasmids and hairpin RNAs with and without ATA was tested by northern blotting and southern blotting. For this purpose cells were transfected as described above and harvested 24, 48, and 72 hr after the beginning of transfection. The growth rate of $2x10^5$ or $5x10^4$ cells, respectively was determined over a period of 10 days by counting cells at 24, 48, 72, 96 hr and 10 days after the beginning of the transfection period. All transfections were performed in triplicate for each time point.

RNA preparation and northern blots

Total RNAs were isolated using RNeasy mini-kits according to the manufacturer's protocol (Qiagen, Hilden, Germany). Probes for northern blots for the detection of PLK1 mRNA were generated by radiolabeling antisense strands for PLK1 and β-actin using 250 μCi of [a-P^{32}]dCTP (6000 Ci/mmol; 1Ci = 37 GBq) for each reaction, 50 μM of each of the other dNTPs, and 10 pmol of either primer PLK1-17-low (5´-TGATGTTGGCACCTGCCTTCAGC-3´), corresponding to position 1533-1554 within the open reading frame of PLK1, or actin-2-low (5´-CATGAGGTAGTCAGTCAGGTC-3´), as described previously (39). The template for the generation of probes corresponds to amino acids 285-497 of PLK1. Northern blotting and hybridizations were carried out as described previously (39). All blots were reprobed with actin probes so that actin-normalized PLK1 mRNA levels could be compared.

For the detection of shRNA/PLK1 a probe was generated by radiolabeling antisense strands for PLK1 using 250 μCi of [a-P32]dCTP (6000 Ci/mmol; 1Ci = 37 GBq) for each reaction, 50 μM of each of the other dNTPs, and

For the detection of shRNA/PLK1 a probe was generated by radiolabeling antisense strands for PLK1 using 250 μCi of [a-P32]dCTP (6000 Ci/mmol; 1Ci = 37 GBq) for each reaction, 50 μM of each of the other dNTPs, and 10 pmol of primer PLK1-150-as (5´-GCAGCAGAGACTTAGGCACAA-3´), corresponding to position 310-330 within the open reading frame of PLK1, as described previously. As template, the entire cDNA of human PLK1 was used. Total mRNA from transfected cells was separated on a 10% polyacrylamid-8M urea gels at 200 V in TBE for 1 hr and transferred to Hybond N+ membranes (Amersham Pharmacia Biotech., Freiburg, Germany) for 1 hr at 200 mA. The blots were prehybridized in QuickHyb® (Stratagene, Amsterdam, The Netherlands) for 20 min. and hybridized at 68°C for 1 hr. Membranes were washed twice in 2xSSC for 15 min at 36°C and exposed to MP Hyperfilms (Amersham Pharmacia Biotech., Freiburg, Germany).

Isolation of DNA from transfected cells, tumor tissue and from murine blood

Total DNA was isolated using QIAamp DNA mini-kits according to the manufacturer's protocol (Qiagen, Hilden, Germany) to determine the amount of plasmid in cell culture, tumors and the blood of nude mice (see below). DNA was separated on 1% agarose gels (100 V, 30 min) and plasmids were detected by ethidium bromide staining using an UV transilluminator.

Southern blot analysis

To determine the effect of ATA on the stability of plasmids, total DNA was isolated and electrophoresed as described above. Gels were stained with ethidium bromide and photographed. To depurinize and denature DNA, gels were incubated 15 min in 0.25 M HCl on a shaker to induce doublestrand breaks and thereafter 30 min under denaturing conditions (1.5 M NaCl and 0.5 M NaOH) on a shaker. To neutralize gels were incubated 2x 15 min in neutralizing solution (1.5 M NaCl, 0.5 M Tris-HCl, pH 7.2, and 1 mM

EDTA, pH 8.0). Then DNA was transferred onto nylon membranes as described for northern blotting analysis. Membranes were dried at room temperature and DNA was fixed on membranes for 5 min on an UV transilluminator. Blots were hybridized as described for northern blot analysis.

Western blot analysis

For western blot analysis cells were lysed and protein concentration determined as described (40). Fifty μg of total protein were separated on a 12% SDS-polyacrylamide gel and were then transferred (85V; 1.5 hr) to ImmobilonTM-P transfer membranes (Millipore, Bedford, MA). Membranes were incubated for 1 hr in 5% nonfat powdered milk in PBS with monoclonal antibodies against PLK1 (1:250) and actin (1:200,000) or with monoclonal (1:100) and actin (1:200,000) followed by incubation with goat anti-mouse serum (1:2000) for 30 min and visualized as described before (41).

As was done for northern blotting experiments, PLK1 protein expression was routinely normalized to actin protein expression levels. The resulting normalized PLK1 protein levels were then presented relative to those in mock-transfected cells.

In northern and western blotting experiments PLK1 and actin expression was quantified using a Kodak gel documentation system (1D 3.5). Integration of signal intensities from scanned autoradiographs was followed by quantitative comparison of PLK1 and actin expression, that is, for each treatment the ratio of PLK1 and actin signals was determined. Values are given in percentage of levels in mock-transfected cells.

Determination of cell proliferation

The number of cells at each time point was determined using a hemacytometer. Cell viability was assessed by trypan blue staining. The

number of mock-treated cells (incubated with normal culture medium without FuGENETM6 or plasmids) after 96 hr was used as a reference. Ratios of plasmid-treated cells and mock-treated cells was determined to obtain the percentage of proliferating cells. Each experiment was performed in triplicate. Means and 95% confidence intervals were determined.

Fluorescence-activated cell sorting analysis

Cell cycle distribution and apoptosis were analyzed using a Becton Dickinson FACScan apparatus. Cells were harvested, washed with PBS, and probed with CycleTESTTM PLUS DNA reagent kit (Becton Dickinson, Heidelberg, Germany) according to the manufacturer's protocol to determine cell cycle distribution. For each transfection (mock-transfected and each plasmid), 30,000 cells were analyzed in triplicate. Percentage of cells in different cell cycle phases was calculated using ModFit LT for Mac. For the detection of apoptotic phenotypes, harvested cells were fixed with ice-cold 70% ethanol, treated for 20 min at 37°C with RNase A (5 μg/mL) and with propidium iodide (50 μg/mL). Subsequent analyses of cell cycle distribution and apoptosis were performed using the CELLQuest software (Becton Dickinson, Heidelberg, Germany).

DNA staining of transfected cells

48 hr after the beginning of transfection cells were washed twice in PBS and fixed for 15 min in icecold methanol. After washing three times in PBS for 10 min DNA was stained with 2-(4-amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI) (Sigma-Aldrich, Taufkirchen, Germany) for 20 min. Cells were examined with a fluorescence microscope (Leica, Wetzlar, Germany) at a magnification of 40x.

Measurement of U6-based shRNA efficacy in nude mouse tumor models

Human cancer xenograft models were established with at least 3 independent groups of 5 athymic nude mice (nu/nu) NMRI 8-10 weeks old.

passaged by a minimum of three consecutive transplantations prior to treatment. Then tumor fragments were implanted subcutaneously in both flanks of the nude mice and treatment with plasmids was started when the tumor reached a volume of 100 mm^3. Treatment was carried out every other day by injection of 500 μl PBS containing 10 μg plasmid and 2 μg ATA (ATA:DNA at a ratio of 1:5) into the tail vein. One group was treated with plasmid expressing shRNA2/PLK1 without ATA, the second group with a combination of pBS/shRNA2/PLK1 and ATA. Another group was treated with pBS/shRNA/PLK1S with or without ATA as control. The mock-treated group received 500 μl PBS every other day. Tumor diameters were determined using a caliper. Volumes were calculated according to the formula $V = \pi/6$ x largest diameter x smallest diameter^2. Experiments were carried out in triplicate, means and 95% CI were calculated. After sacrificing the animals tumors were excised for detection of shRNA/PLK1 using northern blot analysis.

Statistical methods

Each western blot and northern blot experiment was performed three times. Means of normalized (i.e., to actin) signal intensities were calculated. For the determination of proliferation, cell numbers were determined in triplicate at each time point. FACScan analyses were carried out three times for each cell type. Statistical analysis was performed with two-way ANOVA (GraphPad Prism, GraphPad Software, Inc., San Diego, California) to consider random effects of individual gels and different treatments. For two-way ANOVAs all treatment groups were compared to mock-transfected cells. P values and 95% confidence intervals (CI) for the statistical significance of the changes caused by each transfection are given.

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Claims

1. A pharmaceutical composition for suppressing gene expression comprising an effective amount of
1) at least one RNA expression system and
2) a nuclease inhibiting substance,
wherein said RNA expression system contains
a) at least one RNA polymerase specific promoter sequence and under the transcriptional control of said promoter sequence
b) at least one genetic information homologous to said gene to be suppressed
wherein said genetic information under suitable conditions and in the presence of an RNA polymerase is transcribed into interfering RNA.

2. The pharmaceutical composition according to claim 1, wherein said interfering RNA is a siRNA, preferably a shRNA (hairpin), or a short antisense RNA.

3. The pharmaceutical composition according to claim 1 or 2, wherein said RNA expression system is contained on a plasmid or viral vector.

4. The pharmaceutical composition according to claims 1, 2 or 3, wherein the genetic information b) comprises two complementary and inverted sequences (hairpin) which are homologous to said gene to be suppressed.

5. The pharmaceutical composition according to claim 4, wherein each of said two sequences is 15 to 30 nucleotides long.

6. The pharmaceutical composition according to anyone of claims 1 to 5, wherein said sequences are connected by a spacer sequence.

7. The pharmaceutical composition according to claim 6, wherein said spacer sequence contains 3 to 10 nucleotides.

8. The pharmaceutical composition according to anyone of claims 1 to 7, wherein the the genetic information b) contains an RNA polymerase stop signal at the 3' end.

9. The pharmaceutical composition according to anyone of claims 1 to 8, wherein the siRNA is a small hairpin RNA (shRNA).

10. The pharmaceutical composition according to anyone of claims 1 to 9, wherein the nuclease inhibitor is aurin tricarboxylic acid (ATA).

11. The pharmaceutical composition according to anyone of claims 1 to 10, wherein the RNA specific promoter is the U6 promoter or H1 promoter.

12. The pharmaceutical composition according to anyone of the preceding claims, wherein it is formulated for intravenous administrations.

13. The pharmaceutical composition according to claim 12, wherein it is formulated for bolus injection.

14. The pharmaceutical composition according to claims 12 or 13, wherein the active substances are contained in buffered saline solution.

15. The pharmaceutical composition according to anyone of claims 1 to 14, wherein the expression system in contained in an amount suitable for delivery of 0.05 to 0.5 mg/kg body weight of a patient.

16. The pharmaceutical composition according to anyone of claims 1 to 15 which is suitable for suppressing expression of genes which are associated with tumor formation, growth or metastasis.

17. The pharmaceutical composition according to claim 16, wherein it is suitable for suppressing PLK1 or HER2/neu expression.

18. The pharmaceutical composition according to anyone of claims 1 to 15, which is suitable for suppressing expression of viral genes.

19. The pharmaceutical compostition according to claim 18, which is suitable for suppression expression of at least one gene of a virus selected from HIV, Hepatitis viruses, EBV, CMV or Herpes simplex viruses.

20. Use of a pharmaceutical composition according to anyone of claims 1 to 19 for the suppression of expression of undesired gene products.

21. Use according to claim 20, wherein undesired gene products are proteins or nucleic acids associated with tumour formation, growth, maintainance or formation of metastases.

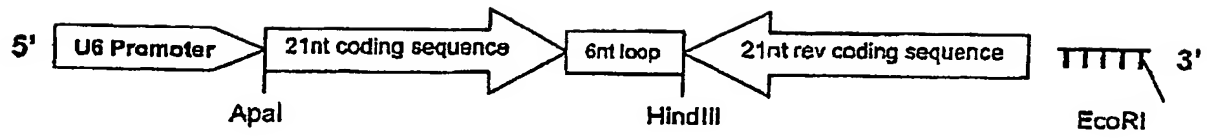
22. Use according to claim 20, wherein undesired gene products are proteins or nucleic acids which are encoded by viruses and are necessary for the viral infectivity or proliferation.

Abstract

The present invention concerns a pharmaceutical composition for suppressing undesired gene expression and especially expression of genes which are overexpressed in patients suffering from certain diseases like cancer disease. Also the expression of viral genes and correspondingly viral infection can be suppressed using the pharmaceutical composition of the present invention. The pharmaceutical composition contains at least one expression system encoding at least one interfering RNA and nuclease inhibitor.

Fig. 1

A



shRNAs:

PLK1 GGGCGGCUUUGCCAAGUGCUU
UUUUCCCGCCGAAACGGUUCACGAA

PLK1S GGGCCCUGUACUAGGUUGCUG
UUUUCCCGGGACAUGAUCCAACGAC

Fig. 2

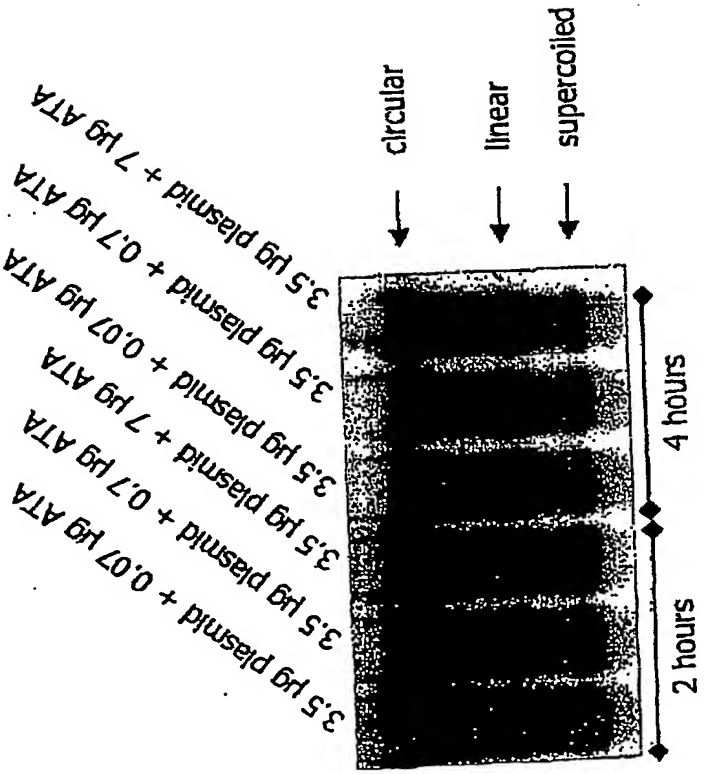
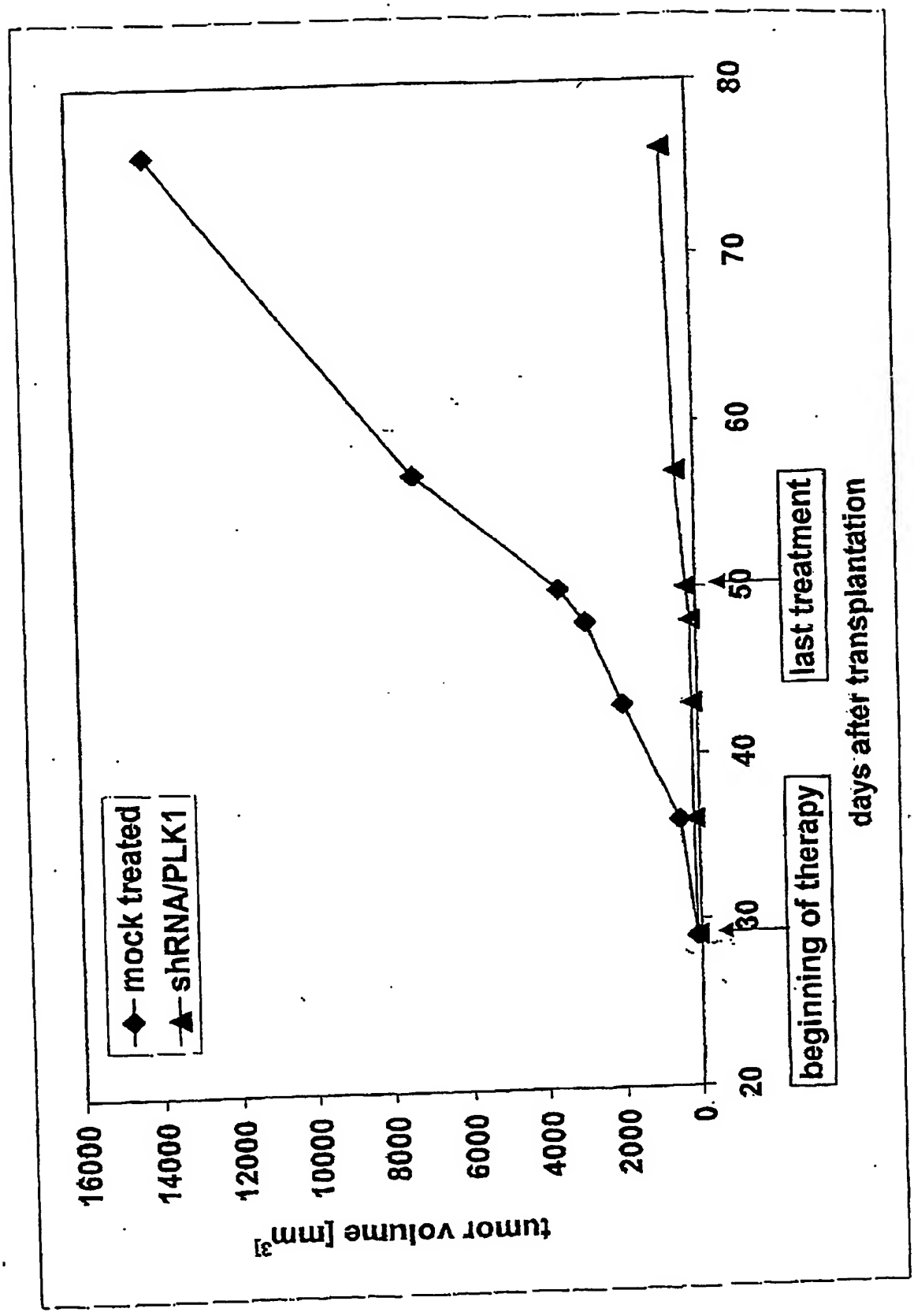


Fig. 3


tumor volume [mm³]
16000
14000
12000
10000
8000
6000
4000
2000
0
20
30
40
50
60
70
80
days after transplantation
beginning of therapy
last treatment
mock treated
shRNA/PLK1